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1. Walton et al., Journal of clinical endocrinology and metabolism. 2001 August. Vol. 86, No. 8, pp. 3675-85.
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Bao-Thuy Nguyen
(571) 272-0824
Remsen 3D51
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The International Standard for Pituitary FSH: Collaborative study of the Standard and of four other purified human FSH preparations of differing molecular composition by bioassays, receptor assays and different immunoassay systems

P. L. Storring and **R. E. Gaines Das**

National Institute for Biological Standards and Control, (WHO International Laboratory for Biological Standards), Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG

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ABSTRACT

The International Standard for Pituitary FSH (IS; in ampoules coded 83/575) was assayed in terms of the Second International Reference Preparation of Human Pituitary FSH and LH for Bioassay (IRP 78/549) by 27 laboratories in 13 countries using bioassays, receptor assays and immunoassays. Estimates of the FSH content of the IS by in-vivo bioassay were homogeneous both within and between laboratories and gave a combined geometric mean (with 95% fiducial limits) of 79·9 (74·6–85·4) i.u./ampoule. Estimates by different in-vitro bioassays and receptor assays were also homogeneous between assays and laboratories, and gave a combined geometric mean (with 95% fiducial limits) of 31·2 (28·8–33·9) i.u./ampoule. However, estimates by the 19 different immunoassay systems were heterogeneous and varied between 5 and 31 i.u./ampoule.

The material in ampoules coded 83/575 was established by the World Health Organization as the International Standard for Pituitary FSH. It was assigned a unitage of 80 i.u./ampoule on the basis of its calibration by in-vivo bioassay, because this assay best identifies and defines the hormone. However, the introduction of the new IS will necessitate the recalibration of immunoassay kits.

FSH 84/530, prepared in the same way as the IS from the same FSH preparation, did not differ significantly from the IS in any of the assay systems studied and appeared to be equally suitable as a standard.

Four highly purified preparations of human FSH (FSH A–D), differing in their isoform compositions and in their in-vivo : in-vitro bioactivity ratios, were also studied. The ranking order of the specific activities of FSH A–D by in-vitro bioassays paralleled their order by receptor assays and the order of their content of FSH isoforms with isoelectric points > 4·5. (Potency estimates of FSH B and C in terms of the IS were greater by receptor assay than by in-vitro bioassay.) The overall ranking order of the specific activities of FSH A–D by immunoassays was different. Contrary to expectation, estimates in terms of the IS of specific activities by immunoassay differed more between preparations than those by in-vitro bioassay or receptor assay.

Differences in specificity between immunoassay systems were demonstrated not only in the calibration of the IS in terms of the crude FSH of IRP 78/549 but also in the comparisons of the highly purified FSH in the IS and FSH A–D. The differences in the immunoreactivities and bioactivities of FSH preparations differing in their isoform compositions greatly complicate the standardization of assays for FSH.

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INTRODUCTION

Immunoassays of follicle-stimulating hormone (FSH) in human plasma have many important applications in clinical medicine and reproductive physiology. Various materials, including the International Reference Preparations (IRP) of pituitary and urinary FSH and luteinizing hormone (LH) for bioassays, have been used as standards for these assays. (Separate standards for FSH and LH from pituitaries and from post-menopausal urine were established as the evidence then available suggested that the gonadotrophins extracted from these two sources differed from each other.) Such standards consisting of crude FSH extracts are not, however, suitable for immunoassay systems which are not entirely specific for FSH.

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The Expert Committee on Biological Standardization of the World Health Organization (WHO ECBS) therefore requested the National Institute for Biological Standards and Control (NIBSC) to obtain highly purified human pituitary FSH for the preparation of an international standard for human pituitary FSH (WHO ECBS, 1975).

A preparation of purified human pituitary FSH was subsequently obtained from a laboratory experienced in its isolation. The purity of a FSH preparation cannot be assessed only on the basis of its physicochemical properties because FSH, like other glycoproteins, is intrinsically heterogeneous (Reichert & Ramsey, 1971; Zaidi, Robertson & Diczfalusy, 1981). Its FSH biological potency therefore becomes an important criterion of purity. This material had an FSH potency of about 5000 i.u./mg by in-vivo bioassay (Steelman & Pohley, 1953) in terms of the First IRP of Human Pituitary FSH and LH for Bioassay (IRP 69/104; Bangham, Berryman, Burger *et al.* 1973; WHO ECBS, 1975). This appeared to be of the same order of potency as that of other highly purified FSH described in the literature. The preparation was therefore ampouled in readiness for its collaborative study (WHO ECBS, 1979). However, the biological potencies reported in the literature for purified FSH are difficult to compare because different standards, often of ovine origin, have been used for these estimates, and because the protein content of the preparations has been determined by different methods. A comprehensive comparison was therefore carried out (by bioassays, immunoassay and isoelectric focusing) of 12 preparations of purified FSH, originating from six laboratories (Storring, Zaidi, Mistry *et al.* 1981; Zaidi, Fröysa & Diczfalusy, 1982). This study indicated that FSH could be purified to yield material with a potency, by in-vivo bioassay in terms of IRP 69/104, of about 15 000 i.u./mg, some threefold higher than that of the FSH which had earlier been ampouled. Such a more potent preparation was therefore obtained and ampouled to become the International Standard for Pituitary FSH (IS).

This paper describes the IS and FSH 84/530 (a batch of ampoules prepared identically from the same material) and their collaborative study by different bioassays and immunoassays. Four preparations of highly purified FSH differing in their isoform compositions and bioactivities were also included in this study to assess the specificity of these assay systems and the effects of different isoforms on FSH standardization.

PARTICIPANTS

The 27 laboratories from 13 countries which took part in the study are listed below. Throughout this report, each laboratory is identified by a number between 1 and 27, but these numbers do not correspond to the order of listing. H. T. Araldi, B. N. Giampaolo and R. O. Cinto, Instituto Nacional de Farmacologia y Bromatologia, Avenida Caseros 2161, 1264 Buenos Aires, Argentina; D. Graham and M. Poulis, Pharmacology Section, National Biological Standards Laboratory, GPO Box 462, Canberra, ACT 2601, Australia; D. M. Robertson and L. Foulds, Department of Anatomy, Monash University, Clayton, Victoria 3168, Australia; B. David, I. R. E., Institut National des Radioelements, B-6220 Fleurus, Belgium; M. R. Sairam and G. N. Bhargavi, Reproduction Research Laboratory, Clinical Research Institute of Montreal, 110 avenue des Pins ouest, Montreal, Quebec H2W 1R7, Canada; Sun Liu-nan and Xu Li-gen, Ministry of Health, National Institute for the Control of Pharmaceutical and Biological Products, Temple of Heaven, Beijing with Chen Kun-ming, Tianjin Medical College, Tianjin, China; H. H. Petersen, Pharmaceutical Laboratories, National Board of Health, 378 Frederikssundsvej, DK-2700 Brønshøj, Denmark; W. Wagner and R. Floren, Mallinckrodt Diagnostica (Germany) GmbH, von Hevesy-Str 1–3, D-6057 Dietzenbach 2, F.R.G.; Y. Combarnous and N. Martinat, Station de Physiologie de la Reproduction, Institut National de la Recherche Agronomique, Centre de Recherches de Tours, 37380 Nouzilly, France; J. Giroux, Département de Pharmacologie-Toxicologie-Cosmétologie, Laboratoire National de la Santé, 14 Rue Ecole de Pharmacie, F-34000 Montpellier, France; T. Ishii, Eiken Immunochemical Laboratory, Eiken Chemical Co Ltd, 26–20, 5-Chome, Oji, Kita-ku, Tokyo 114, Japan; S. A. Khan and E. Diczfalusy, Reproductive Endocrinology Research Unit, Karolinska Hospital, Box 60500, S-104 01 Stockholm 60, Sweden; L Larsson, Y. Gunnarsson and R. Stroemberg, Pharmacia Diagnostics AB, S-751 82 Uppsala, Sweden; L. Wide, Department of Clinical Chemistry, University Hospital, S-75014 Uppsala, Sweden; A. M. G. Bosch and W. J. H. M. Stevens, Diagnostics Research and Development Laboratories, Organon, PO Box 20, 5340 BH Oss, The Netherlands; F. H. de Jong and S. W. J. Lamberts, Departments of Biochemistry and Medicine, Erasmus Universiteit Rotterdam, Postbus 1738, 3000 DR Rotterdam, The Netherlands; J. G. Loeber, National Institute of Public Health and Environmental Hygiene, PO Box 1, 3720 BA Bilthoven, The Netherlands; K. M. Ferguson and S. L. Jeffcoate, Endocrine Department, Chelsea Hospital for Women, Dovehouse Street, London SW3 6LT, U.K.; I. D. Gilham and J. F. Wright, Boots-Celltech Diagnostics Ltd, 240 Bath Road, Slough SL1 4ET, U.K.; B. M. Hobson, Centre for Reproductive Biology, University of Edinburgh, 37 Chalmers

Street, Edinburgh EH3 9EW, U.K.; S. S. Lynch and W. R. Butt, Department of Clinical Endocrinology, The Birmingham and Midland Hospital for Women, Showell Green Lane, Sparkhill, Birmingham B11 4HL, U.K.; P. L. Storring and Y. G. Mistry, Division of Endocrinology, National Institute for Biological Standards and Control, Hampstead, London NW3 6RB, U.K.; S. A. Aroesty, Diagnostic Products Corporation, 5700 West 96th Street, Los Angeles, CA 90045, U.S.A.; R. Behling and R. T. Dworschack, Diagnostics Division, Abbott Laboratories, Abbott Park, IL 60064, U.S.A.; M. Baran, H. Kumar and B. T. Monfiston, Becton Dickinson Immunodiagnostics, 13 Mountain View Avenue, Orangeburg, NY 10962-1294, U.S.A.; L. E. Reichert Jr, P. M. Sluss and A. L. Schneyer, Department of Biochemistry, The Albany Medical College of Union University, Albany, NY 12208, U.S.A.; M. J. Stella and A. E. Leek, Serono Diagnostics, 100 Longwater Circle, Norwell, MA 02061, U.S.A.

MATERIALS AND METHODS

Materials

Second IRP of Pituitary FSH and LH (ICSH), Human, for Bioassay (IRP 78/549)
Each ampoule (coded 78/549) contains 0·5 mg of an extract of human pituitaries, and has a defined unitage per ampoule of 10 i.u. FSH activity and 25 i.u. LH activity (WHO ECBS, 1981). The IRP 78/549 was prepared from the same batch of master ampoules (coded 69/133) and by the same procedure as the IRP 69/104 (Bangham *et al.* 1973). Its FSH and LH bioactivities did not differ significantly from those of IRP 69/104 (Storring, Khan, Mistry & Diczfalusy, 1988).

International Standard for Pituitary FSH (IS)
This consists of a batch of ampoules (coded 83/575) containing highly purified human pituitary FSH; another batch of ampoules of the same material (coded 84/530) was prepared identically (FSH 84/530).

Bulk FSH. Some 40 mg FSH was purified, and characterized by immunoassay, by Professor W. R. Butt, Dr. S. S. Lynch and their colleagues (Birmingham), and generously donated to WHO. This consisted of three separate batches (coded CPDS sfa 3–5, CPDS sfa 6–10 and CPDS sfa 11,16,17) prepared by the same procedures from acetone-dried human pituitaries (Butt & Lynch, 1974). They were received at NIBSC as frozen solutions in 200 mmol sodium chloride/l–10 mmol sodium phosphate/l (pH 7·2). Table 1 shows details of their characterization.

Distribution into ampoules. The IS and FSH 84/530 were put into ampoules under identical conditions in November 1983 and March 1984 respectively. For each batch of ampoules, 11·5 ml CPDS sfa 3–5, 17 ml CPDS sfa 6–10 and 13·5 ml CPDS sfa 11,16,17 were passed through a (0·45 μm) membrane filter (Millex-HA, Millipore SA, 67-Molsheim, France), pooled, and diluted to a concentration of 8·34 μg/ml (in 15·4 mmol sodium chloride/l, containing 0·2% (w/v) heat-treated repurified human plasma albumin (batch AK11, Lister Institute, Elstree, Herts, U.K.) and 1% (w/v) mannitol). This solution was distributed into ampoules as approximately 0·5 ml aliquots. The ampoule contents were freeze-dried, secondarily desiccated and sealed under nitrogen (Campbell, 1974; WHO ECBS, 1978). The IS consisted of 3872 ampoules and the batch FSH 84/530 of 4045

TABLE 1. Characteristics of the bulk FSH used for the International Standard for Pituitary FSH and FSH 84/530

	Batch		
	CPDS sfa 3–5	CPDS sfa 6–10	CPDS sfa 11,16,17
Volume of batch (ml)	24	35	27·5
Protein concentration (μg/ml)[a]	437	437	435
FSH bioactivity (i.u./mg)[b];	16 900;	15 100;	17 209;
95% fiducial limits	14 000–20 500	12 900–17 700	13 929–21 262
(no. of assays)	(2)	(2)	(2)
FSH immunoreactivity (i.u./mg)[c]	3328	5142	4044
LH immunoreactivity (i.u./mg)[d]	124	190	209
TSH immunoreactivity (m.i.u./mg)[e]	59	144	144

[a]From the absorbance of the solutions in 10 mmol sodium phosphate/l–200 mmol sodium chloride/l (pH 7·2), assuming that the absorbance at 280 nm of a 1% (w/w) solution of FSH in a 1 cm light-path is 10.
[b]By in-vivo bioassay (Steelman & Pohley, 1953) in terms of IRP 78/549.
[c]By immunoassay in terms of IRP 69/104.
[d]By immunoassay in terms of the First IRP of Human Pituitary LH for Immunoassay.
[e]By immunoassay in terms of the First IRP of TSH for Immunoassay (WHO ECBS, 1975).

ampoules. The mean weight of solution in each of 80 or more weighed ampoules was 0·503 g for the IS and 0·506 g for FSH 84/530 with a range (as % of the mean) of 0·78% and 0·47% respectively. Each ampoule of these two batches contains about 4·17 μg FSH, 5 mg mannitol, 1 mg human plasma albumin and 0·6 mg sodium chloride.

Activity of ampoule contents. Preliminary estimates by in-vivo bioassay (Steelman & Pohley, 1953) and immunoassay indicated that the FSH activity of the bulk had been retained during its distribution into ampoules and that the IS was adequately stable.

The LH activity of the IS (with 95% fiducial limits) in terms of IRP 78/549 was estimated to be 1·27 (0·448–2·67) i.u./ampoule by in-vivo bioassay (Parlow, 1961) and 0·344 (0·304–0·389) i.u./ampoule by an in-vitro bioassay (Van Damme, Robertson & Diczfalusy, 1974); the LH activity of FSH 84/530 was estimated to be 0·414 (0·359–0·471) i.u./ampoule by the same in-vitro bioassay.

Purified LH and thyroid-stimulating hormone (TSH)
Purified LH consisted of the IRP of Human Pituitary LH for Immunoassay (in ampoules coded 68/40; Storring, Bangham, Cotes *et al.* 1978). Purified TSH consisted of a preparation in ampoules coded 81/502 (Gaines Das & Bristow, 1985).

Human sera
One pool of serum from healthy women menstruating normally and one from healthy postmenopausal women were distributed into ampoules as 1 ml aliquots and freeze-dried. These two batches of ampoules were coded serum Y and X respectively.

Coded preparations of purified FSH
Six preparations of FSH in ampoules coded FSH A–F were also included in the study. FSH F was a coded duplicate of the IS, and FSH E consisted of ampoules of the IS which had been kept at 37 °C for 355 days.

FSH A, B, C, and D were prepared from materials earlier donated to WHO as candidate standards by Drs W. R. Butt, P. J. Lowry and A. F. Parlow and by Kabi Diagnostica AB (Stockholm, Sweden) – although not in this order. Their activities and isoform compositions had been shown to differ (Storring *et al.* 1981; Zaidi *et al.* 1982). For this study, solutions of FSH B–D were distributed into ampoules and freeze-dried by methods similar to those used for the IS; FSH A had been put into ampoules earlier. In addition to FSH, each ampoule contained approximately 1 mg human plasma albumin with 5 mg lactose (FSH A) or mannitol (FSH B, C and D). Details of these preparations and of their earlier characterization are given in Table 2.

Methods

Design of the study
Participants were asked to contribute assays which were, as far as possible, specific for FSH. These were to include bioassays and immunoassays, especially if the latter were unique to the laboratory of the participant with respect to the antiserum used. Each participant was asked to carry out at least two independent assays by each method used for each preparation tested. Participants were asked to examine preparations using at least three dose levels in the linear part of the dose–response curve in order to assess the linearity and parallelism of dose–response lines.

TABLE 2. Characteristics of purified FSH preparations FSH A–D

	FSH A	FSH B	FSH C	FSH D
Codes in earlier studies[a,b]	77/532[a] D[b]	B	A	C
FSH content (μg/ampoule)[c]	2·32	7·82	9·62	4·59
FSH potencies as i.u./mg[d] by in-vivo bioassay[e] (95% fiducial limits)	4650 (4080–5310)	5640 (4670–6810)	8260 (7160–9530)	12 700 (10 100–16 000)
FSH potencies as i.u./mg[d] by in-vitro bioassay[f] (95% fiducial limits)	7740 (7390–8160)	4100 (3860–4350)	3660 (3120–4180)	8150 (7890–8390)
In-vivo : in-vitro bioactivity	0·60	1·4	2·3	1·6
Proportion of bioactive FSH with pI < 4·5[g]	77·3	78·9	95·3	89·2

[a]Storring *et al.* (1981).
[b]Zaidi *et al.* (1982).
[c]Based on the absorbance of solutions of the bulks, assuming that the absorbance at 280 nm of a 1% (w/w) solution of FSH in a 1 cm light-path is 10 and that the ampoules contained the amount formulated.
[d]From the data of Storring *et al.* (1981) in terms of the IRP 69/104.
[e]Steelman & Pohley (1953).
[f]Van Damme, Robertson, Marana *et al.* (1979).
[g]From the data of Zaidi *et al.* (1982) calculated in terms of the total bioactivity recovered in the different fractions.

TABLE 3. Details of FSH in-vitro bioassays and receptor assays used in this study

	Laboratory	Geometric mean cross-reactivity (% w/w)[f] LH 68/40	TSH 81/502
In-vitro bioassays			
Oestradiol production by rat Sertoli cells[a]	1	0·34	0·29
	15	NT	NT
Plasminogen activator secretion by rat granulosa cells[b]	24	NT	NT
Receptor assays			
Binding of ^{125}I-labelled human FSH by calf or bull testis membrane[c]	4	0·67	0·38
Binding of ^{125}I-labelled human FSH by calf testis membrane[d]	21	0·53	0·40
Binding of ^{125}I-labelled rat FSH by rat testicular homogenate[e]	24	NT	NT
Binding of ^{125}I-labelled ovine FSH by pig granulosa membrane	25	0·67	0·29

[a]Van Damme *et al.* (1979).
[b]Beers & Strickland (1978).
[c]Cheng (1975).
[d]Branca, Sluss, Smith & Reichert (1985).
[e]Combarnous, Guillou & Martinat (1984).
[f]Expressed as % (w/w) of the International Standard for Pituitary FSH (IS) having the equivalent effect, assuming that the contents per ampoule were: 4·17 μg FSH for the IS; 11·6 μg LH for LH 68/40; and 2 μg TSH for TSH 81/502.
NT, not tested.

Participants offering in-vitro bioassays, receptor assays or immunoassays were asked to include, as far as possible, all of the 13 preparations listed under Materials. In each assay every preparation was to be included at as many dilutions as practicable, to provide information about the dose–response curves. If practicable, a different batch of labelled FSH was to be used for each immunoassay and receptor assay, and in addition a different batch of the receptor preparation for each receptor assay.

Participants were asked to provide full results of their assays, including all raw data. They were also asked for their own calculated estimates of potency.

Assays contributed to the study

Estimates by the in-vivo (human chorionic gonadotrophin (hCG)-augmented ovarian weight gain) bioassay (Steelman & Pohley, 1953) were contributed by the laboratories shown in Table 5. Details of the in-vitro bioassays and receptor assays are given in Table 3 and of the immunoassay systems in Table 4. Laboratory 17 contributed results from two immunoassay systems which have been denoted by 17A and 17B.

Statistical analysis

For each assay, log dose–response relationships were examined for linearity. If necessary, assay responses were transformed to give a more nearly linear log dose–(transformed) response relationship. The logarithm of the ratio of ovary weight to body weight was taken as the response for in-vivo bioassays. Logit transformation (as described by Gaines Das & Cotes, 1979) was used for immunoassays and for about half of the receptor assays and in-vitro bioassays.

An analysis of variance was computed for each assay and the linearity and parallelism of the log dose–response lines were assessed. Responses were analysed as multiple parallel-line assays to determine log potency estimates. Log potency estimates for preparations (notably LH and TSH) with log dose–response lines which were markedly non-parallel with those of the standard(s) were determined from the responses nearest the 50% response of the assay system. Analysis was repeated omitting such log dose–response lines to obtain potency estimates for the other preparations.

For the in-vivo bioassays, a statistical weight was determined for each log potency estimate as the reciprocal of the variance of the log potency estimate. For each laboratory carrying out immunoassays, receptor assays and in-vitro bioassays, an average statistical weight for the mean log potency estimates by each type of assay was based on the within-assay variation (as measured by the deviation of the log potency of the coded duplicate from the log of 100%) and the between-assay variation of the individual log potency estimates.

TABLE 4. Details of FSH immunoassay methods used in this study

	Laboratory	Type of antiserum (code if known)	Geometric mean cross-reactivity (% w/w)[d] LH 68/40	TSH 81/502
One-site assays[a]				
Radioimmunoassays using double-antibody precipitation	1	(M93/2)	0·37	0·52
	2		0·64	0·77
	4	(M93/2)	0·30	0·004
	5	(M93/2)	0·13	0·29
	16		<2·16	<1·25
	17A[b]		9·20	2·15
	17B		4·86	1·42
	18		0·16	0·48
	21	Batch 4 from US National Hormone and Pituitary Program	0·33	1·31
	22		1·05	0·94
Radioimmunoassays using double-antibody solid-phase precipitation	3		0·01	0·06
	8	(M93/2)	0·32	0·25
	11		0·28	0·25
	13	(M93/2)	0·11	0·42
Radioimmunoassay using adsorption to solid-phase for separation	25	Anti-ovine FSH	0·83	0·42
Radioimmunosorbent assay using solid-phase separation[c]	15	Attached to solid-phase	0·20	0·21
Two-site assays				
Immunoradiometric assay using sucrose-layering for separation	9	^{125}I-labelled anti-hFSH monoclonal + anti-hFSH polyclonal attached to solid-phase	0·04	0·06
Immunoradiometric assay using precipitation with a third antibody	26	Two anti-hFSH polyclonals – one of which was labelled with ^{125}I	1·22	2·44
Double-antibody sandwich enzyme immunoassay using solid-phase separation	10	Anti-hFSH polyclonal attached to solid-phase + anti-hFSH polyclonal attached to horse-radish peroxidase	0·40	9·15
	23	Anti-hFSH-α subunit monoclonal attached to solid-phase + anti-hFSH-β subunit monoclonal attached to horse-radish peroxidase	2·24	2·90

[a]These assays used ^{125}I-labelled human FSH as tracer and polyclonal antisera, which were directed against human FSH except for that of laboratory 25.
[b]This assay system included an antiserum to LH for simultaneous assay of FSH and LH.
[c]Wide, Nillius, Gemzell & Roos (1973).
[d]Expressed as % (w/w) of the International Standard for Pituitary FSH (IS) having the equivalent effect, assuming that the contents per ampoule were: 4·17 μg FSH for the IS; 11·6 μg LH for LH 68/40 and 2 μg TSH for TSH 81/502.

Log potency estimates were examined for heterogeneity by chi-squared test. Differences among laboratories and among different types of assay were also assessed by analysis of variance of (unweighted) log potency estimates. Homogeneous potency estimates were combined as weighted geometric means with fiducial intervals based on the sum of weights of the estimates combined. Heterogeneous potency estimates were combined as unweighted geometric means with fiducial intervals determined using the variance of the log potency estimates combined.

RESULTS

Calibration of the IS and FSH 84/530 in terms of IRP 78/549

In-vivo bioassays

Log dose–response lines for these three preparations did not deviate significantly from parallelism, apart from the limited exceptions noted in Table 5. In terms of IRP 78/549, estimates of the FSH content per ampoule of each of the IS and FSH 84/530 were homogeneous both within and between laboratories

TABLE 5. Estimates of the FSH content of the International Standard for Pituitary FSH (IS) and FSH 84/530 by in-vivo bioassay[a] in terms of IRP 78/549. The number of estimates are given in parentheses

	FSH content (i.u./ampoule)			
	IS		FSH 84/530	
	Geometric mean	95% fiducial limits	Geometric mean	95% fiducial limits
Laboratory				
6	91·0 (1)	65·5–182·1	61·9 (2)	49·4–77·7
7	91·1 (2)	73·9–112·3	—	—
12	77·6 (1)[b]	55·1–109·0	83·1 (1)[c]	59·2–117·1
14	83·3 (2)	63·0–85·2	74·7 (1)[c]	59·8–92·5
19	78·4 (2)	66·8–91·2	76·1 (2)	64·7–89·5
20	81·0 (3)	71·3–91·9	84·6 (2)[b]	73·2–97·9
27	78·2 (2)	63·2–96·8	84·7 (2)	67·2–106·8
Weighted geometric mean for all labs	79·9 (13)	74·6–85·4	77·8 (10)	71·9–84·1
Unweighted geometric mean for all labs	—	—	71·6 (12)[d]	62·7–81·7

[a]Augmented ovarian weight gain assay (Steelman & Pohley, 1953) using rats, except for laboratory 12 which used mice according to the method of Brown (1955).
[b]Results from another assay omitted due to significant ($P < 0·05$) non-parallelism.
[c]Results from another assay omitted due to significant contribution to overall heterogeneity.
[d]Including two estimates from laboratories 12 and 14 which contributed significantly to overall heterogeneity.

with two exceptions (Fig. 1 and Table 5). The weighted geometric mean of estimates of the FSH content (with 95% fiducial limits) was 79·9 (74·6–85·4) i.u./ampoule for the IS and 77·8 (71·9–84·1) i.u./ampoule for FSH 84/530. If the two estimates which contributed excessively to the value of the chi-square for heterogeneity were included, the unweighted geometric mean of the FSH content (with 95% fiducial limits) of FSH 84/530 was 71·6 (62·7–81·7) i.u./ampoule. Statistical analysis did not suggest any difference between the FSH contained in ampoules of the IS and FSH 84/530.

In-vitro bioassays

Log dose–response lines for the three preparations did not show consistent significant deviations from linearity or parallelism. In terms of IRP 78/549, estimates of the FSH content per ampoule for the IS were similar in the three laboratories; however the estimates for FSH F (identical to the IS) and FSH 84/530 were somewhat larger in laboratory 15 (Table 6 and Fig. 1). The weighted geometric mean of estimates of the FSH content (with 95% fiducial limits) was 32·8 (28·1–38·2) i.u./ampoule for the IS and 34·8 (29·8–40·6) i.u./ampoule for FSH 84/530. These estimates were less than half those obtained by in-vivo bioassay.

Receptor assays

Log dose–response lines for these three preparations did not deviate consistently from linearity or parallelism except in laboratory 21, where the log dose–response line for IRP 78/549 was consistently steeper than those for the IS or FSH 84/530. In terms of IRP 78/549, estimates of the FSH content per ampoule of each of the IS and FSH 84/530 were similar in these four assay systems (Table 6 and Fig. 1). The weighted geometric mean of estimates of the FSH content (with 95% fiducial limits) was 30·6 (27·8–33·7) i.u./ampoule for the IS and 31·1 (28·2–34·3) i.u./ampoule for FSH 84/530. These estimates were similar to those obtained by in-vitro bioassay.

Immunoassays

Log dose–logit response lines for IRP 78/549, the IS and FSH 84/530 did not show consistent significant deviations from linearity. Their slopes were generally similar to one another, except that the slope for IRP 78/549 was consistently steep in laboratory 18 and consistently flat in laboratories 10, 22, 23 and in four of the five laboratories (1, 5, 8 and 13) using antiserum M93/2.

Comparison of the IS and FSH F (identical to the IS; Table 7) provides a direct estimate of the minimum variability inherent in these assay systems. For these data the pooled within-assay variance was similar to the pooled between-assay variance (omitting laboratory 1, the ratio of within : between assay variances was 1·32). The reason for the consistent difference in

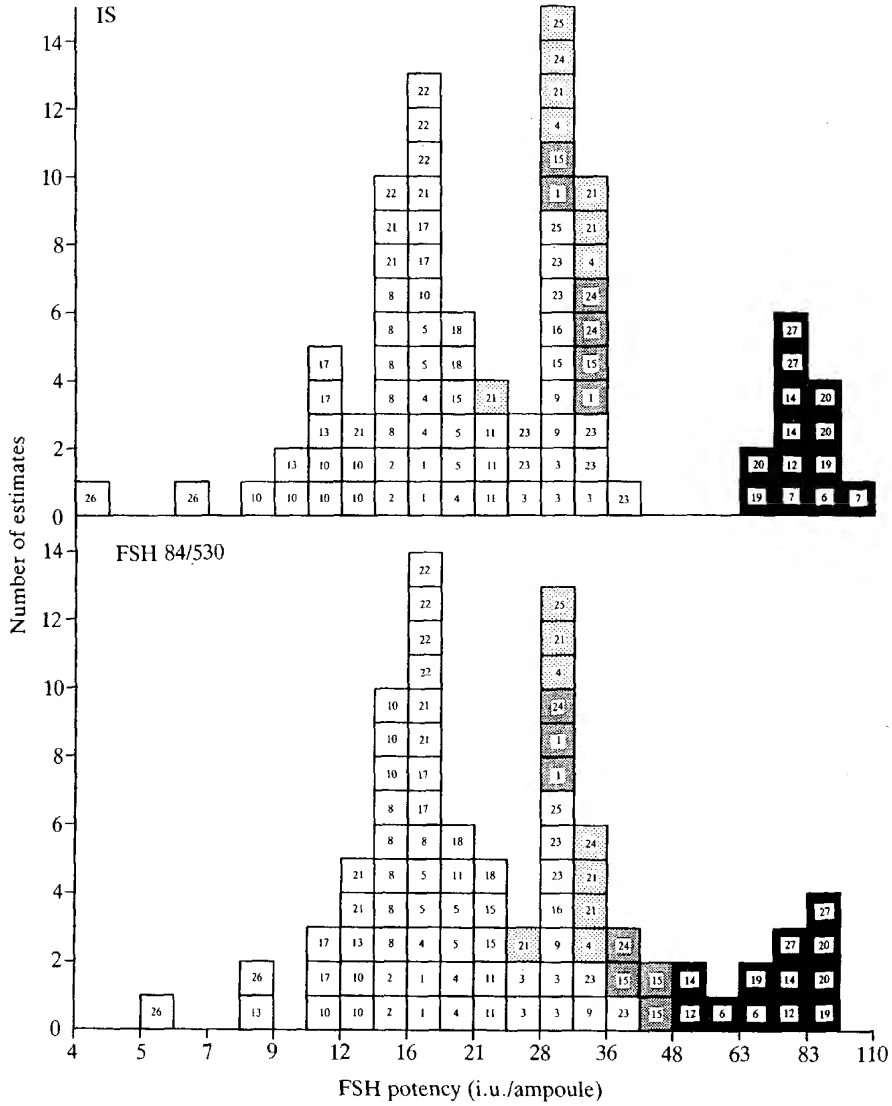

FIGURE 1. Frequency distribution of log potencies of the International Standard for Pituitary FSH (IS) and of FSH 84/530 in terms of IRP 78/549 by in-vivo bioassays (solid squares), in-vitro bioassays (heavily stippled squares), receptor assays (lightly stippled squares) and immunoassays (open squares) in different laboratories (identified by numbers).

values obtained for FSH F and the IS in laboratory 1 is not known. Estimates for FSH F in terms of the IS (omitting those from laboratory 1) were not significantly more variable between different laboratories than between assays in the same laboratories (ratio of variances 1·51).

Estimates for the IS and FSH 84/530 in terms of IRP 78/549 did not differ significantly (Table 7). Furthermore, comparisons within and between laboratories for estimates of FSH 84/530 in terms of the IS gave results similar to those obtained for estimates of FSH F (identical to the IS).

Estimates of the FSH content of the IS and FSH 84/530 in terms of IRP 78/549 were very similar within laboratories; however, there were marked differences in estimates between laboratories (Table 7 and Fig. 1). Estimates for the IS, FSH F or FSH 84/530 in terms of IRP 78/549 (range of laboratory means 5 to 32 i.u./ampoule, or 30% to 180% of the overall mean) were some 14 times more variable between laboratories

TABLE 6. Estimates of the FSH content of the International Standard for Pituitary FSH (IS), FSH F (a coded duplicate of the IS), FSH 84/530 and ampoules of the IS kept at 37 °C for 355 days by in-vitro bioassays and receptor assays in terms of IRP 78/549. The number of estimates are given in parentheses

		Geometric mean FSH content (i.u./ampoule)			
	Laboratory	IS	FSH F	FSH 84/530	IS kept at 37 °C
In-vitro bioassay					
	1 (2)	31·0	25·2	28·9	29·8
	15	33·2 (2)	38·9 (2)	42·9 (3)	—
	24	35·4 (2)	26·9 (1)	33·3 (2)	—
	Geometric mean for all labs[a]	32·8	29·8[b]	34·8	29·8
	95% fiducial limits	28·1–38·2	—	29·8–40·6	—
Receptor assay					
	4 (2)	32·0	29·7	31·3	31·6
	21 (4)	29·0	31·0	31·1	31·4
	24 (1)	31·1	27·5	32·5	—
	25 (1)	28·9	32·9	28·9	29·7
	Geometric mean for all labs[c]	30·6	30·2	31·1	31·2
	95% fiducial limits	27·8–33·7	27·4–33·3	28·2–34·3	28·2–34·6

[a]Weighted geometric mean of laboratory means with statistical weights of 350, 350 and 200 for laboratories 1, 15 and 24 respectively.
[b]Unweighted geometric mean of heterogeneous laboratory means; fiducial limits not calculated.
[c]Weighted geometric mean of laboratory means using statistical weights of 1000, 600, 300 and 300 for estimates from laboratories 4, 21, 24 and 25 respectively.

than estimates for FSH F or FSH 84/530 in terms of the IS (range some 90% to 120% of the mean). At least three distinct groups of estimates were apparent: laboratories 3, 9, 16, 23 and 25 with estimates between 28 and 31 i.u./ampoule; laboratory 26 with exceptionally low estimates; and the remaining 14 laboratories with estimates between 10 and 23 i.u./ampoule. This latter group was itself heterogeneous; it included a subgroup of estimates from laboratories 1, 4, 5, 8 and 13, each using antiserum M93/2, which was, however, as heterogeneous as the entire group. The group of five laboratories (3, 9, 16, 23 and 25) giving the highest estimates included the two assay systems (laboratories 3 and 9) with the lowest cross-reactivities for LH and TSH and the only two-site assay systems (laboratories 9 and 23) which used monoclonal antibodies. The estimates from these five laboratories together with estimates by in-vitro bioassays and receptor assays formed a homogeneous group with geometric mean (and 95% fiducial limits) of 30·0 (28·5–31·6) i.u./ampoule for the IS and 30·3 (28·7–31·9) i.u./ampoule for FSH 84/530. Estimates from the two-site assays considered as a group were no more homogeneous than all estimates.

When ranked in terms of their cross-reactivity with LH and TSH (Table 4), the immunoassay systems with smaller cross-reactivities tended to give higher estimates for the FSH content of the IS or FSH 84/530 in terms of IRP 78/549; but this relationship was not significant. The cross-reactivities with TSH of three of the four two-site assays were greater than any of those of the one-site assays (Table 4).

Accelerated thermal degradation studies of the IS

The FSH content of ampoules of the IS kept at 37 °C for 120 days was estimated in laboratory 27 by in-vivo bioassay in terms of IRP 78/549. The combined potency estimate (with 95% fiducial limits) from two assays was 72·3 (57·2–91·5) i.u./ampoule. This was not significantly different from estimates in the same laboratory of the FSH content of the IS by in-vivo bioassay (Table 5). There were no detectable differences between the slopes of log dose–response lines for IRP 78/549, the IS or samples of the IS kept at 37 °C.

Ampoules of the IS kept at 37 °C for 355 days (coded FSH E) were compared with ampoules of the IS stored at −20 °C (normal conditions) using in-vitro bioassays, receptor assays and immunoassays (Tables 6 and 7). No consistent differences were detected between slopes of the log dose–response lines for the IS or FSH E in these assay systems. Analysis of variance for estimates by each type of assay showed no significant difference between estimates of potency in terms of the IS for FSH E and for FSH F (identical to the IS). Estimates for FSH E and F did not differ

TABLE 7. Estimates of the FSH content of the International Standard for Pituitary FSH (IS), FSH F (a coded duplicate of the IS), FSH 84/530 and ampoules of the IS kept at 37 °C for 355 days by immunoassays in terms of IRP 78/549. The number of estimates are given in parentheses

	Geometric mean FSH content (i.u./ampoule)			
	IS	FSH F	FSH 84/530	IS kept at 37 °C
Laboratory and assay				
1 (2)	16·6	24·9	17·0	24·6
2 (2)	14·6	15·0	14·7	14·3
3 (4)	29·8	30·5	27·7	27·7
4 (3)	18·0	19·9	18·3	21·6
5	18·2 (4)	20·4 (2)	17·9 (4)	20·3 (2)
8 (6)	15·1	15·7	15·3	15·2
9 (2)	29·4	29·5	31·4	28·8
10	11·6 (7)	12·4 (7)	13·7 (6)	13·5 (7)
11	22·2 (3)	21·7 (2)	21·2 (3)	22·1 (2)
13 (2)	10·3	9·2	10·1	9·4
15 (2)	23·2	22·4	21·8	—
16	30·4	—	28·0	—
17A (2)	10·8	—	10·8	10·6
17B (2)	17·0	—	17·2	16·3
18 (2)	20·2	20·0	20·4	19·6
21 (4)	15·2	15·3	15·1	15·6
22	16·3 (4)	16·8 (2)	16·7 (4)	17·0 (2)
23	30·7 (8)	—	32·2 (4)	—
25 (1)	27·7	26·5	27·7	24·6
26 (2)	5·5	6·4	6·5	—
Unweighted geometric mean for all labs	17·6	17·8	17·9	18·0
95% fiducial limits	14·4–21·7	14·3–22·3	14·8–21·6	15·1–21·4

significantly among different laboratories except that estimates by immunoassay in laboratory 1 for each of E and F were some 50% larger than expected.

Immunoassay estimates of the FSH content of serum samples

Serum samples were assayed with relatively fewer dilutions, and responses to these were often near the extremes of the response range. Consequently comparisons of the log dose–response lines were not reliable. Nevertheless there was a tendency for serum Y in laboratory 5 and both X and Y in laboratories 10 and 13 to have flatter log dose–response lines than the IS. In laboratories 5 and 13 (which both used antiserum M93/2), log dose–response lines for Y also tended to be flatter than those for IRP 78/549.

Estimates for the FSH content of the serum samples, in terms of either IRP 78/549 or the IS, showed considerable variability among laboratories (Table 8). In terms of either standard, the variability of estimates for FSH in serum Y (from normally menstruating women) was greater than that in serum X (from post-menopausal women). For each of serum X and serum Y, this variability was about two times greater for estimates in terms of the IS than for those in terms of IRP 78/549. However, omitting results from laboratories 9, 23 and 26 substantially reduced the variability among laboratories for estimates of the FSH content of X in terms of the IS to a value similar to that for estimates in terms of IRP 78/549, and likewise for estimates of the FSH content of Y. No significantly better agreement was obtained between laboratory estimates if the serum samples were compared one in terms of the other.

Comparison of the highly purified FSH of the IS and of FSH A–D

In-vitro bioassay and receptor assay

There were no consistent significant differences among the slopes of the log dose–response lines for FSH A–D, the IS and for IRP 78/549 in the in-vitro bioassay or receptor assay systems, except in the receptor assays of laboratory 21 where the line for IRP 78/549 was consistently steeper.

In terms of the IS, estimates for each of FSH A–D (Table 9 and Fig. 2) were statistically homogeneous by χ^2 test among these seven assay systems. But analysis of variance showed significant ($P<0·05$) differences

TABLE 8. Estimates of the FSH content of serum X and Y by immunoassays in terms of IRP 78/549 and the International Standard for Pituitary FSH (IS). The number of estimates are given in parentheses

	Geometric mean FSH content (m.i.u. IRP 78/549 /ampoule of serum)		**Geometric mean FSH content** (ampoules of IS × 10^3 /ampoule of serum)	
	Serum X	Serum Y	Serum X	Serum Y
Laboratory and assay				
2 (2)	68	11·8	4·66	0·81
3 (4)	80	7·5	2·70	0·25
4 (3)	68	7·3	3·79	0·40
5 (4)	59	9·2	3·25	0·51
8	40 (5)	5·3 (5)	2·73 (4)	0·35 (4)
9 (2)	39	0·5	1·32	0·01
10 (7)	36	9·8	3·09	0·84
11	50 (3)	5·4 (2)	2·24 (3)	0·25 (2)
13 (2)	50	5·5	4·85	0·54
15 (2)	47	4·6	2·03	0·20
16 (1)	59	7·2	1·93	0·24
17A (2)	29	2·9	2·67	0·27
17B (2)	35	3·5	2·16	0·21
18 (1)	39	4·7	1·91	0·23
21 (2)	33	6·5	2·15	0·43
22 (2)	40	4·2	2·45	0·26
23 (4)	55	5·4	1·49	0·17
26 (2)	44	5·6	8·10	1·02
Unweighted geometric mean for all labs	47	5·1	2·68	0·30
95% fiducial limits	41–54	3·7–7·2	2·15–3·34	0·19–0·46

TABLE 9. Potency estimates by in-vitro bioassays and by receptor assays of the purified FSH preparations A, B, C and D in terms of the International Standard for Pituitary FSH (IS), expressed as % (w/w) on the basis of nominal ampoule contents. The number of estimates are given in parentheses

		Geometric mean FSH potency in terms of the IS (%; w/w)			
	Laboratory	FSH A	FSH B	FSH C	FSH D
In-vitro bioassay					
	1	73·7 (4)	70·9 (2)	33·8 (4)	77·2 (2)
	15 (2)	89·9	71·4	31·6	64·5
	24 (2)	80·9	80·0	31·6	74·5
	Weighted geometric mean for all labs	82·7	73·0	32·5	71·8
	95% fiducial limits	70·1–95·2	62·4–84·8	27·7–38·1	60·9–83·6
Receptor assay					
	4 (2)	84·5	75·7	35·1	76·3
	21 (4)	97·0	93·3	40·3	85·4
	24 (1)	68·3	79·4	39·0	71·8
	25 (1)	89·9	94·9	46·0	78·1
	Weighted geometric mean for all labs	86·3	83·2	37·3	78·1
	95% fiducial limits	77·3–95·2	75·7–91·7	34·7–42·5	70·9–86·3

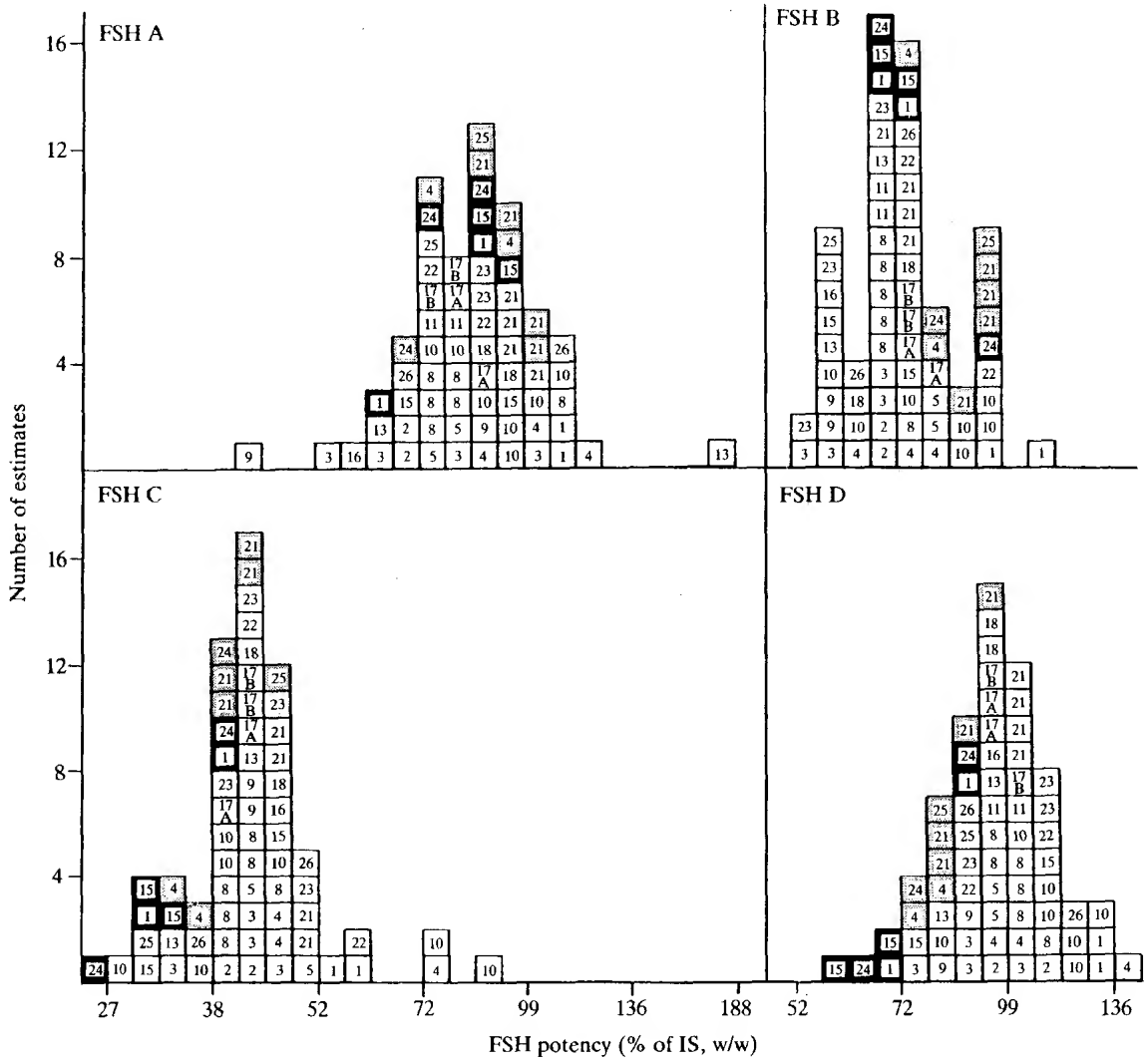

FIGURE 2. Frequency distribution of log potencies of FSH A–D in terms of the International Standard for Pituitary FSH (IS) by in-vitro bioassays (solid squares), receptor assays (stippled squares) and immunoassays (open squares) in different laboratories (identified by numbers).

TABLE 10. Potency estimates by in-vitro bioassays, by receptor assays and by immunoassays of the purified FSH preparations A, B, C and D in terms of IRP 78/549

		Geometric mean FSH potency for all laboratories in terms of IRP 78/549 as i.u./ampoule (95% fiducial limits)			
	No. of laboratories	FSH A	FSH B	FSH C	FSH D
Assay method					
In-vitro bioassay[a]	3	15 (13–18)	45 (28–52)	26 (22–30)	26 (22–30)
Receptor assay[a]	4	15 (13–16)	48 (43–53)	27 (25–30)	26 (24–29)
Immunoassay[b]	20	8·08 (6·76–9·65)	23·2 (19·1–28·1)	17·2 (13·9–21·4)	19·1 (15·7–23·1)

[a]Weighted geometric mean estimates.
[b]Unweighted geometric mean estimates.

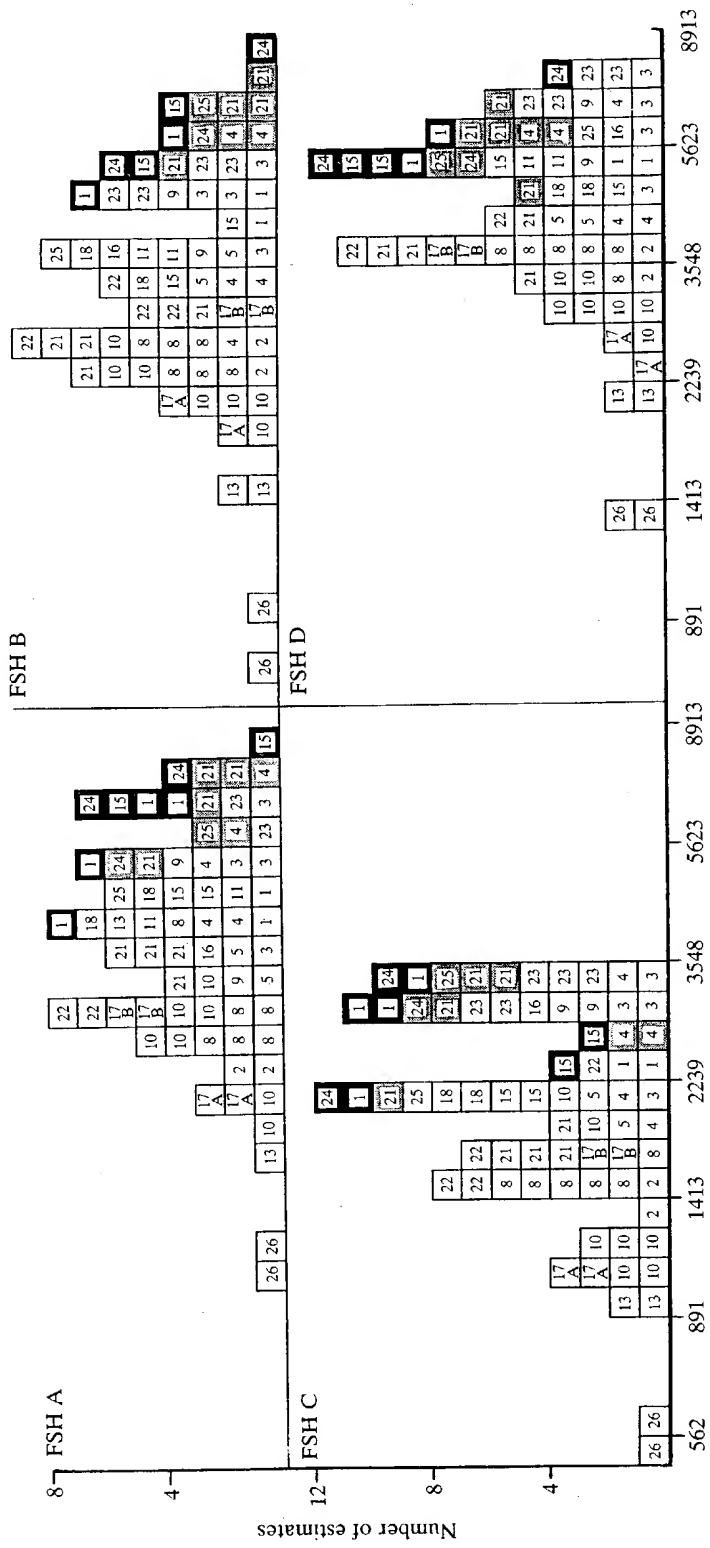

FIGURE 3. Frequency distribution of log potencies of FSH A–D in terms of the IRP 78/549 by in-vitro bioassays (solid squares), receptor assays (stippled squares) and immunoassays (open squares) in different laboratories (identified by numbers).

TABLE 11. Potency estimates by immunoassays of the purified FSH preparations A, B, C and D in terms of the International Standard for Pituitary FSH (IS), expressed as % (w/w) on the basis of nominal ampoule contents. The number of estimates are given in parentheses

	Geometric mean FSH potency in terms of the IS (%; w/w)			
	FSH A	FSH B	FSH C	FSH D
Laboratory and assay				
1 (2)	108*	104*	55·9*	131*
2 (2)	68·3*	69·3	40·3	104
3 (4)	73·7	64·0	39·4	88·1
4 (3)	102	72·0	54·2	112
5 (2)	77·3	78·9	45·5	94·5
8 (6)	82·7	69·8	41·2	101
9 (2)	61·1*	59·7*	42·0	83·6
10 (7)	91·6	80·5	46·8	111
11 (2)	79·1	67·7	—	99·0
13 (2)	110*	62·4	38·1	86·3
15 (2)	82·7	66·1	37·7	92·7
16 (1)	59·3*	60·3*	46·4	94·5
17A (2)	82·7	76·8	40·3	95·4
17B (2)	79·1	75·2	42·0	97·2
18 (2)	89·9	69·8	44·2	97·2
21 (4)	98·8*	72·5	47·3	103
22	82·7 (2)	83·2 (4)	48·6 (4)	99·9 (2)
23	84·5 (2)	61·3 (3)	42·9 (4)	101 (3)
25 (1)	71·9	56·5*	30·8*	89·9
26 (2)	88·1	67·2	42·9	103
Unweighted geometric mean for all labs	82·5	70·1	43·1	98·6
95% fiducial limits	76·2–89·3	65·7–75·9	40·4–46·0	94·1–103
Weighted geometric mean for all labs except estimates marked (*)	81·2	72·3	42·4	97·0
95% fiducial limits	78·9–83·7	70·3–73·8	41·3–43·7	94·6–99·7

*Estimates contributed excessively to chi-square for heterogeneity.

between estimates by in-vitro bioassay and receptor assay for FSH B and C. The weighted geometric mean estimates by receptor assay for each of the four preparations in terms of the IS were higher than those by in-vitro bioassay, that for FSH C was some 18% larger and that for FSH B some 10% larger.

In terms of IRP 78/549, estimates for each of FSH A–D (Fig. 3) were also homogeneous among these seven assay systems, and analysis of variance showed no significant difference between estimates by in-vitro bioassay and receptor assay. The combined estimates of FSH content for all laboratories are shown in Table 10.

The FSH specific activities of FSH A–D (i.e. activity per unit mass based on their nominal ampoule contents) estimated by these two assay systems are shown in Table 9 and Figs 2 and 3. Their ranking order and that of the IS by both types of assay and in terms of both the IS and IRP 78/549 was: IS > FSH A > FSH B > FSH D > FSH C. In contrast to the finding for the immunoassays (below), there were no great differences in the ranking order of the estimated specific activities between any of these seven assay systems. In all assay systems, the IS was most potent and only FSH C was markedly different (less active) in its activity from the other preparations.

Immunoassay

There were no consistent significant differences among the slopes of the log dose–logit response lines for FSH A–D and the IS, except that the line for A was consistently steeper in laboratory 18.

Variability of estimates. In terms of the IS, estimates of the FSH content of each of A–D were consistent between laboratories with a limited number of exceptions (Table 11 and Fig. 2). Omitting laboratory 1, estimates for D were homogeneous and the variation

among estimates from different laboratories was similar to that of the coded duplicate of the IS (data not shown). Estimates for each of A, B and C were more variable among different laboratories than estimates for D, and estimates for A were most variable of all. Omitting a limited number of laboratories in addition to laboratory 1 markedly reduced the variability among estimates for each of these preparations and the remaining estimates were homogeneous (Table 11).

Examination of the estimates which contributed excessively to the between-laboratory variability suggested differences between the specificities of some of these immunoassay systems (Table 11 and Fig. 2). For example, estimates by laboratory 21 were significantly higher than those by laboratory 2 for FSH A and less so for FSH C, but not for FSH B or D. Also, estimates by laboratory 9 were lower than estimates by laboratory 21 for FSH B and D, but not significantly so for FSH A and C.

In terms of IRP 78/549, estimates of the FSH content for each of A–D were not consistent among different laboratories (Fig. 3). Between-laboratory variability for estimates in terms of IRP 78/549 was five or more times greater than between-laboratory variability of estimates in terms of the IS, except for FSH A (three times). Unweighted geometric mean estimates for all laboratories are shown in Table 10.

Specific activities. The FSH specific activities of FSH A–D estimated by the different immunoassay systems are shown in Table 11 and Figs 2 and 3. Their ranking order and that of the IS for all laboratories, when assayed both in terms of the IS and of IRP 78/549, was IS > FSH D > FSH A > FSH B > FSH C. This differed from the ranking order by in-vitro bioassays and by receptor assays in that D was higher than A and B.

In terms of the IS, the specific activities of the IS and FSH D were similar, but were significantly higher than those of each of FSH A, B and C, which in turn differed significantly from one another (Table 11 and Fig. 2). Estimates of the specific activities for FSH A, B and D by immunoassay differed more than those by in-vitro bioassay or receptor assay. Although FSH C was least potent in all assay systems, the ranking order of the specific activities of the IS, FSH A, B and D differed in some immunoassay systems from the overall ranking order. Thus laboratories 2, 16 and 22 and the five assay systems using antiserum M93/2 showed such differences in ranking order. However, these differences in ranking were not significant because of the variability of estimates within assay systems.

In terms of the IS, immunoassay estimates for A and B did not differ significantly from those by in-vitro bioassays; but immunoassay estimates for the FSH content of C and D were significantly larger than estimates by in-vitro bioassay (Tables 9 and 11, Fig. 2).

DISCUSSION

Calibration of the IS and FSH 84/530 in terms of IRP 78/549

In terms of IRP 78/549, estimates of the FSH content of the IS by in-vivo bioassay were homogeneous both within and between laboratories (Table 5 and Fig. 1). Estimates of the FSH content of the IS by the different in-vitro bioassay and receptor assay methods were also homogeneous between assays and laboratories (Table 6 and Fig. 1). On the other hand, FSH immunoassay estimates of the IS varied between 5 and 31 i.u./ampoule (Table 7 and Fig. 1). This indicated considerable differences in specificity between these immunoassay systems, and that as a group they were unsuitable for the calibration of the IS.

The higher potency of the IS by in-vivo than by in-vitro bioassay (ratio 2·4) might be due to the greater plasma survival, and therefore more prolonged action *in vivo*, of the FSH in the IS than of the FSH in IRP 78/549. FSH D, prepared in the same laboratory as the FSH of the IS, was also more potent by in-vivo than by in-vitro bioassay (Table 2). This preparation contained a higher proportion of bioactive FSH with an isoelectric point (pI) <4·5 than IRP 69/104 which is essentially identical to IRP 78/549 (Table 2; Zaidi *et al.* 1982). The presence of these more acidic FSH isoforms in FSH preparations has been shown to correlate with the ratios of their in-vivo : in-vitro FSH bioactivities (Storring *et al.* 1981, Storring, Zaidi, Mistry *et al.* 1982; Zaidi *et al.* 1982; Wide & Hobson, 1983, 1986; Wide, 1985), and also with their FSH plasma survival (Wide, 1986; Wide & Hobson, 1986). These more acidic isoforms appear to represent FSH molecules with a greater degree of sialylation (Wide, 1982, 1985) and thereby enhanced plasma survival (Morell, Gregoriadis, Scheinberg *et al.* 1971).

The higher estimates for the FSH potency of the IS by bioassay than by immunoassay appear to reflect the presence of some immunoreactive FSH in the crude FSH of IRP 78/549 which is devoid of bioactivity, as suggested by the electrofocusing studies of the essentially identical IRP 69/104 (Zaidi *et al.* 1981).

The activities of the different mixtures of FSH isoforms in FSH A–D

Human (pituitary) FSH exists as a mixture of (bioactive) molecular species of differing charge (Reichert & Ramsey, 1971; Zaidi *et al.* 1981). The mixture of these isoforms synthesized and secreted varies with the physiological status of the subject (Wide, 1982, 1985; Wide & Hobson, 1983). Purified preparations of pituitary FSH of differing isoform compositions

(Zaidi *et al.* 1982) were found to differ in their in-vivo and in-vitro bioactivities and in their activities in one immunoassay system (Storring *et al.* 1981). Four of these preparations (FSH A–D; Table 2) were included in this study.

The higher potency estimates of FSH A–D, in terms of the IS, by receptor assays than by in-vitro bioassays (statistically significant for FSH B and C) are probably a reflection of the differences in the carbohydrate structures of the FSH isoforms. Thus, the removal of carbohydrate from FSH reduced its bioactivity drastically but not its receptor-binding activity (Calvo, Keutmann, Bergert & Ryan, 1986). No such differences between in-vitro bioassay and receptor assay estimates were observed with IRP 78/549 as standard. This is probably because IRP 78/549 differs from the IS in containing a wider spectrum of bioactive FSH isoforms and/or FSH-like molecules with receptor-binding activity but without bioactivity.

The ranking order of the specific activities of FSH A–D estimated by both in-vitro bioassays and receptor assays (FSH A > FSH B > FSH D > FSH C; Table 9) was the inverse of their order of content of FSH isoforms with pI <4·5 (Table 2). This suggested that these more acidic FSH isoforms, although high in in-vivo bioactivity, were relatively less potent *in vitro*. In their earlier study these four FSH preparations and three others also showed an inverse correlation ($r=0{\cdot}876$; $P<0{\cdot}05$) between their specific activity by in-vitro bioassay and their contents of bioactive FSH with pI <4·5 (Storring *et al.* 1981; Zaidi *et al.* 1982). In that study (Storring *et al.* 1981), FSH D showed a higher specific bioactivity than FSH A–C; but at that time FSH B, C and D had not yet been distributed into ampoules. The subsequent distribution of FSH C and D into ampoules appears to have reduced their specific bioactivities. Wide & Hobson (1986) have also shown that the more acidic isoforms of FSH are of relatively lower in-vitro FSH bioactivity.

The specific immunoreactivities of FSH A–D differed significantly from one another (Table 11 and Fig. 2), suggesting a difference between the immunoreactivities of FSH isoforms. But the specific immunoreactivities of FSH A–D did not correlate with their content of isoforms with pI <4·5.

The data of this study also indicated that immunoassay systems differed in their specificities for different isoforms of FSH. Thus the estimated ranking orders for the specific activities of the IS and FSH A–D differed between immunoassay systems (Table 11 and Fig. 2). Moreover, estimates of potency between some immunoassay systems differed more for some preparations than for others. Immunoassay estimates in terms of the IS were least variable for FSH D, which was isolated in the same laboratory as the FSH of the IS and had a specific immunoreactivity closest to that of the IS, and immunoassay estimates were most variable for FSH A, which contained least FSH bioactivity with pI <4·5 and had the lowest potency by in-vivo bioassay and the lowest in-vivo : in-vitro bioassay potency ratio (Table 2).

The finding that highly purified FSH preparations of differing molecular compositions differ in their in-vitro bioactivity (and receptor-binding activity) and immunoreactivity has been extended by this collaborative study from one assay system of each type (Storring *et al.* 1981; Zaidi *et al.* 1982) to several, including many immunoassays in current clinical use. Estimates of specific activities (i.e. activity per unit mass of hormone) obtained in this and the earlier study provide a more definitive characterization of FSH than estimates only of ratios of bioactivity : immunoreactivity (e.g. Zaidi *et al.* 1981; Wide & Hobson, 1983; Wang, Dahl, Leung *et al.* 1987). Such ratios often introduce the implicit or explicit assumption that the specific immunoreactivities of different forms of FSH are constant and so give a good approximation of mass. But this study has shown that the specific immunoreactivities of FSH A, B and D differed more than their specific in-vitro bioactivities or receptor-binding activities.

These differences in the immunoreactivities and bioactivities between FSH isoforms greatly complicate the standardization of assays for FSH in specimens differing in their contents of its isoforms. The greater variability of immunoassay estimates for FSH in the serum (Y) from normally menstruating women than in the serum (X) from post-menopausal women (Table 8) may be a reflection of this, since such sera have been shown to differ in their isoform compositions (Wide, 1985). The results of this and earlier (Storring *et al.* 1981; Wide & Hobson, 1983) studies showed a better correlation of the immunoreactivities of different preparations of FSH with their in-vitro bioactivities and receptor-binding activities than with their in-vivo bioactivities. This may be because the structural differences between FSH isoforms, and particularly of their carbohydrate moieties, affect pharmacokinetics and so in-vivo bioactivities more than in-vitro bioactivities and immunoreactivities (Wide & Hobson, 1986).

Establishment of the International Standard for Pituitary FSH

Suitability

The WHO has recommended that standards for immunoassays (which do not themselves measure function) should preferably consist of highly purified hormone, because such hormone preparations (a) can be identified as the hormone on the basis of high

specific bioactivity of physicochemically homogeneous moieties, and (b) may diminish discontinuities of unitage resulting from transitions between standards of differing degrees of purity (WHO ECBS, 1975). More recently, the WHO has recommended that such a standard for the natural form of the hormone should consist of material with the highest potency in the in-vivo bioassay system generally recognized by the scientific community as defining that hormone (WHO ECBS, 1982). This also provides a criterion of purity (i.e. freedom from material without that hormone activity). The FSH used to prepare the IS and FSH 84/530 had a biological potency by in-vivo bioassay (Tables 1 and 5) which was at least as high as that of other available preparations (Storring *et al.* 1981). It was also very potent by in-vitro bioassay, receptor assay and immunoassay (Tables 1 and 6).

The IS was also free from significant contamination with the structurally related LH and TSH. Thus immunoassay estimates of the bulk FSH suggested that its TSH content was less than 1% (w/w), assuming a potency of 20 i.u./mg for pure TSH (Table 1). LH immunoassay estimates of the bulk FSH (Table 1), in-vivo bioassay estimates of the IS and in-vitro bioassay estimates of the IS and FSH 84/530 all suggested that the FSH of the IS contained less than 3% (w/w) of LH. This was based on the assumption that the potency for pure LH estimated by the same assays and in terms of the same standard is 10 000 i.u./mg (Storring *et al.* 1982).

The IS appeared to be adequately stable when stored under normal conditions (at −20 °C in the dark), since there was no detectable loss of FSH activity in ampoules kept at 37 °C for about 1 year. FSH 84/530 probably has a similar stability since it was prepared in the same way as the IS.

The IS also appeared to be suitable to serve as a standard for the assay of FSH in clinical samples. Thus for most immunoassay systems in this study, the variability of estimates of the FSH content in the two sera was no greater in terms of the IS as standard than in terms of IRP 78/549.

The variability of FSH immunoassay estimates of the purified preparations FSH A–D by these immunoassay systems was considerably less in terms of the IS as standard than in terms of IRP 78/549.

The suitability of the FSH preparation in the ampoules coded 83/575 to serve as the International Standard was therefore agreed by the participants in the collaborative study.

Establishment and assignment of unitage

The WHO therefore established the material in ampoules coded 83/575 as the IS for Pituitary FSH, and assigned to it a unitage of 80 i.u./ampoule on the basis of its calibration by in-vivo bioassay (WHO ECBS, 1987).

This assignment of unitage was made with the agreement of the majority of participants. It was based on the recommendations of the WHO that such standards be calibrated by bioassay (WHO ECBS, 1969), and that the classical in-vivo bioassay system, generally recognized by the scientific community, best identifies and defines the hormone (WHO ECBS, 1982). Thus in the in-vivo bioassay, the target cells for the hormone are *in situ* and their responsiveness is regulated by the homeostatic control mechanisms of the organism. This ensures that the specificity of a particular in-vivo bioassay is very similar between laboratories. Hence the good agreement in this study between in-vivo bioassay estimates of different laboratories. On the other hand, the preparation of cells or receptors for in-vitro bioassay systems is complex and difficult to control. The specificities of in-vitro bioassays may therefore differ between laboratories. Furthermore the specificity of in-vivo bioassays tends to be of a higher order than that of in-vitro bioassays (Storring, 1989). This is because the in-vivo bioassay involves processes over and above those acting *in vitro*, and these processes are influenced by additional specific structural features of the hormone.

A discontinuity of unitage for most of the immunoassay systems in this study would also have resulted if the assignment of unitage had been based on the calibration by in-vitro bioassays and receptor assays. A valid calibration of the IS by immunoassay was not possible because of the sixfold range of estimates by immunoassay.

The WHO ECBS (1987) recognized that the introduction of the new IS, with a defined potency of 80 i.u./ampoule, would necessitate the recalibration of immunoassay kits, as occurred when the IRP of hCG was established (WHO ECBS, 1979). Because immunoassays of FSH are widely used clinically, and critical decisions depend upon their results, the Committee recommended that manufacturers should state clearly with which standard their kits were calibrated, and that calibration against the new IS should be adopted as rapidly as possible.

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